

TITLE

REARRANGED SQUAMOUS CELL CARCINOMA ANTIGEN GENES

PRIORITY INFORMATION

5 This application is a continuation of PCT patent application serial number PCT/SE02/00512 filed March 15, 2002, which claims priority to Swedish Patent Application No. 0100938-0 filed March 15, 2001 all of which are incorporated herein in their entirety.

BACKGROUND OF THE INVENTION

10 1. Field of Invention

The present invention relates to a fusion gene found in squamous cell carcinomas, detection of the rearrangement and monoclonal antibodies specific for SCCA1, SCCA1/A2, SCCA2/A1 and SCCA2.

15 2. Description of the Prior Art

Squamous cell carcinoma antigen (SCCA) is a serological marker for squamous cell carcinomas (SCC) of the uterine cervix, lung, head and neck, vulva, and esophagus (1, 2). It was originally purified from the TA-4 complex from human cervical squamous cell carcinoma, with a molecular weight of 42-48 kDa (1, 3). The antigen consists of more
20 than 10 proteins and iso-electric focusing of the antigen reveals two subfractions, an acidic (pI<6.25) and a neutral (pI ≥6.25) isoform (4). The difference in molecular weight is probably due to modification (5).

Cloning of the cDNA of SCCA shows that it belongs to the family of serine protease
25 inhibitors (serpins) (6). Further cloning of the genomic region on chromosome 18q21.3 reveals two tandemly arrayed genes (7). The more telomeric one, the original SCCA, was designated SCCA1, whereas the more centromeric one was designated SCCA2 (Figure 1A). They both contain eight exons and the putative intron-exon boundaries, splice sites, initiation codons, and terminal codons are identical. They are 98% identical at the
30 nucleotide level (Figure 2) and 92% identical at the amino acid level (Figure 3). The deduced pI value shows that the neutral isoform is coded by SCCA1, and the acidic isoform by SCCA2. Alternatively spliced variant mRNA from both the genes have been found resulting in proteins 52 and 21 amino acids shorter (5).

In humans the serpins map to one of two chromosomal clusters. PI6, PI9 and ELNAH2 map to 6p25, whereas PI8, Bomapin, PAI2, SCCA1, SCCA2, Headpin and Maspin map to 18q21.3 (Figure 1A)(7-12). These clusters are supposed to have arisen via two independent interchromosomal duplications and several rounds of intrachromosomal duplications (9). The chromosome region 18q has often been reported as a region with high frequency of rearrangements (9, 13-16). The targets and functions of serpins are not fully understood. For most, the primary functions are regulation of proteolytic events associated with coagulation, fibrinolysis, apoptosis and inflammation, but alternative functions such as hormone transport and blood pressure regulation have been reported (17-24).

Although SCCA1 and SCCA2 are nearly identical they differ in their reactive site loops (Figure 2 and 3). SCCA1 inhibits the papain-like cysteine proteinases cathepsin S, K, and L (25, 26) while SCCA2 inhibits the chymotrypsin-like serine proteinases cathepsin G and mast cell chymase (27). Studies of the reactive site loop (RSL) of SCCA1 show that the RSL is essential for cysteine proteinase inhibition (28). The variable portion of the RSL dictates the specificity of the target proteinases shown by RSL swap mutants of SCCA1 and SCCA2 and single mutants (28, 29). It is likely that serpins utilize a common RSL-dependent mechanism to inhibit both serine and cysteine proteinases.

The biological role of SCCA1 and SCCA2 are not fully understood. They are considered to be inhibitory serpins. Data suggest that SCCA1 is involved in apoptosis and expression makes cancer cells resistant to several killing mechanisms by inhibition of apoptosis (30). The role of SCCA2 expression in cancer cells is still unclear. In normal tissue SCCA antigen may have some specific role during epidermal maturation (5).

Recent studies using discriminatory monoclonal antibodies and polymerase chain reaction (PCR) have shown that both SCCA1 and SCCA2 are expressed in the suprabasal layers of the stratified squamous epithelium of the tongue, tonsil, esophagus, uterine cervix and vagina, Hassall's corpuscles of the thymus, some area of the skin and in the stratified columnar epithelium of the conducting airways (31). In squamous cell carcinomas of the lung and head and neck, SCCA1 and SCCA2 were co-expressed in moderately and well-differentiated tumors. In contrast to previous studies using nondiscriminatory antibodies, these data show that there were no differential expression between SCCA1 and SCCA2 in normal and malignant tissue. Previous results have shown that SCCA2 was only detected

at the peripheral parts of the tumor (32). This discrepancy may be due to differences between immunohistochemical techniques and antibody specificities (31). It has been reported that false positive results may often be caused by contamination with saliva or sweat during assay procedure (1). Cataltepe et al. suggest that the SCCAs in saliva are derived from the squamous epithelial cells lining mucosal surfaces of the upper digestive tract (31).

Normally, SCCA1 and SCCA2 are detected in the cytoplasm of squamous epithelial cells (31), but not in the circulation (33). The antigen, which appears in the serum of patients with SCC, may be a function of SCCA-over-production by tumor cells and their normal turn over (34). It has been reported that the SCCA detected in serum by using antibody radioimmuno-assay or RT-PCR is mainly SCCA2 (1, 35, 36) but other studies using PCR indicate that both antigens can be amplified and detected in patient samples (37).

Serum concentrations present in patients with SCC are correlated to the clinical stage and to the degree of histological differentiation of the tumor (1). For cervical cancer several studies show a correlation between the pretreatment values and the clinical outcome (1, 38-43). Studies also show a correlation between high SCCA levels and tumor volume. Recurrence or progressive disease could be detected several months before clinical evidence (39). Similar results are seen for squamous cell carcinomas of the lung, vulva, head and neck and esophagus (1, 2, 44, and 45). In all these studies, they have measured the total SCCA level. Recently a new sELISA was developed using discriminating antibodies for SCCA1 and SCCA2 (33).

SUMMARY OF THE INVENTION

The present invention provides the detection of a fusion gene consisting of SCCA1 and SCCA2. This fusion gene has now been found in SCC cell-lines of different origin (cervix, lung and pharynx). The invention also provides methods for establishment of specific immunological reagents for determination/detection of the fusion protein.

One fusion protein is defined by the following amino acid sequence **(SEQ ID NO: 1)**

MNSLSEANTK FMFDLFQQFR KSKENNIFYS PISITSALGM VLLGAKDNTA QQIKKVLHFD
QVTENTTGKA ATYHVDRSGN VHHQFQKLLTE FNKSTDAYE LKIANKLFGE KTYLFLQEYL
DAIKKFYQTS VESVDFANAP EESRKKINSW VESQTNEKIK NLIPEGNIGS NTTLVLVNAI

YFKGQWEKKF NKEDTKEEFK WPNKNTYKSI QMMRQYTSFH FASLEDVQAK VLEIPYKGKD
 LSMIVLLPNE IDGLQKLEEK LTAEKLMEWT SLQNMRETCV DLHLPRFKME ESYDLKDTLR
 TMGMVNIENG DADLSGMTWS HGLSVSKVLH KAFVEVTEEG VEAAAATAVV VVELSSPSTN
 EEFCNHPFL FFIRQNKTNS ILFYGRFSSP

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based upon the DNA sequence **(SEQ ID NO: 2)**

ATGAATTCAC TCAGTGAAGC CAACACCAAG TTCATGTTCTG ACCTGTTCCA ACAGTTCAGA
 AAATCAAAAG AGAACAACAT CTTCTATTCC CCTATCAGCA TCACATCAGC ATTAGGGATG
 10 GTCCTCTTAG GAGCCAAAGA CAACACTGCA CAACAGATTA AGAAGGTTCT TCACTTTGAT
 CAAGTCACAG AGAACACCAC AGGAAAAGCT GCAACATATC ATGTTGATAG GTCAGGAAAT
 GTTCATCACC AGTTTCAAAA GCTTCTGACT GAATTCAACA AATTCCACTGA TGCATATGAG
 CTGAAGATCG CCAACAAGCT CTTCGGAGAA AAAACGTATC TATTTTTACA GGAATATTTA
 GATGCCATCA AGAAATTTTA CCAGACCAGT GTGGAATCTG TTGATTTTGC AAATGCTCCA
 15 GAAGAAAGTC GAAAGAAGAT TAACTCCTGG GTGGAAGTC AAACGAATGA AAAAATTTAA
 AACCTAATTC CTGAAGGTAA TATTGGCAGC AATACCACAT TGGTTCTTGT GAACGCAATC
 TATTTCAAAG GGCAGTGGGA GAAGAAATTT AATAAAGAAG ATACTAAAGA GGAAAAATTT
 TGGCCAAACA AGAATACATA CAAGTCCATA CAGATGATGA GGCAATACAC ATCTTTTCAT
 TTTGCCTCGC TGGAGGATGT ACAGGCCAAG GTCCTGGAAA TACCATACAA AGGCAAAGAT
 20 CTAAGCATGA TTGTGTTGCT GCCAAATGAA ATCGATGGTC TCCAGAAGCT TGAAGAGAAA
 CTCACTGCTG AGAAATTGAT GGAATGGACA AGTTTGCAGA ATATGAGAGA GACATGTGTC
 GATTTACACT TACCTCGGTT CAAAATGGAA GAGAGCTATG ACCTCAAGGA CACGTTGAGA
 ACCATGGGAA TGGTGAATAT CTTCAATGGG GATGCAGACC TCTCAGGCAT GACCTGGAGC
 CACGGTCTCT CAGTATCTAA AGTCCTACAC AAGGCCTTTG TGGAGGTCAC TGAGGAGGGA
 25 GTGGAAGCTG CAGCTGCCAC CGCTGTAGTA GTAGTCGAAT TATCATCTCC TTCAACTAAT
 GAAGAGTTCT GTTGTAATCA CCCTTTCCTA TTCTTCATAA GGCAAAATAA GACCAACAGC
 ATCCTCTTCT ATGGCAGATT CTCATCCCCA TAGATGCAAT TAGTGTGTCA CT

BRIEF DESCRIPTION OF THE DRAWINGS

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Figs. 1A-1C show the chromosome 18 rearrangement.

Fig. 2 shows the alignment of the coding DNA regions, exon 2-8 of SCCA1 and
 SCCA2. Intron positions are indicated as -Ix-. Differences between the genes are
 35 indicated in bold. The regions coding for reactive site loops are shown in lower-case
 letters. Underlining shows primer (Table 1) positions.

Fig. 3 shows the alignment of protein sequences of SCCA1 and SCCA2. Intron positions are indicated with dotted lines. Differences between the proteins are underlined. Boxes show the reactive site loops.

Fig 4 shows nucleotide coding DNA region, exon 2-8 of the rearranged SCCA1/SCCA2. Sequences derived from SCCA1 are shown in normal style while sequences derived from SCCA2 are shown in bold. Intron positions are indicated as -Ix-. Differences between the genes are underlined. The region coding for reactive site loop is shown in lower-case letters.

Fig. 5 shows the protein sequence of the SCCA1/SCCA2 fusion protein. Amino acids derived from SCCA1 are shown in normal letters. Amino acids derived from SCCA2 are shown in bold letters. Intron positions are indicated with dotted lines. Differences between the proteins are underlined. The reactive site loop is marked with a box.

Fig. 6 is a graph showing the titer of pAB to SCC antigen.

Fig. 7 is a graph showing the reactivity of established hybridomas with different SCC antigens.

Fig. 8 shows the complex-binding analysis of SCCA1/A2 fusion protein. Lane A: SCCA1/A2, Lane B: SCCA1/A2 incubated with Cathepsin G, Lane C: SCCA1/A2 incubated with Cathepsin L. The complex of SCCA1/A2 and Cathepsin G is indicated by an arrow. Molecular weight marker is indicated.

Fig. 9 is a Southern blot analysis of genomic DNA digested with PstI and hybridized with probe I. Lane A: RPMI2650 containing the SCCA1/SCCA2 fusion gene, Lane B: Normal DNA. Aberrant bands are indicated with arrows. Molecular weight marker is indicated.

DETAILED DESCRIPTION OF THE INVENTION

The fusion gene (Figure 4) was found by sequencing cDNA from SCC cell lines.

<u>Cell line</u>	<u>Origin</u>	<u>SCCA1</u>	<u>SCCA2</u>
CaSki	Cervix	normal	A1/A2
C4I	Cervix	normal	normal
A549	Lung	N.A.	A1/A2
5 CaLu3	Lung	normal	normal
SkMES	Lung	normal	normal
RPMI2650	Pharynx	N.A.	A1/A2

According to the sequence swift from SCCA1 to SCCA2, the DNA breakpoint would be in
 10 intron 7 (Figure 2). The gene should consequently be controlled via the promoter region
 of SCCA1 but producing a protein with SCCA2-specificity.

The fusion genes are cloned and kept as plasmid-constructs as well as transformed into
 different E. coli strains.

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A plasmid, pGEX6P-3 SCCA1/A2, containing the fusion gene has been deposited with
 European Collection of Cell Cultures on the 14th of March, 2001, under deposition
 number ECACC 01031315.

20 Fusion protein has been produced and complex binding studies show substrate binding of
 the fusion gene to Cathepsin G but not to Cathepsin L.

The fusion gene can be detected by Southern blot analysis of tumor DNA. The fusion gene
 can also be detected by PCR analysis as well as by cDNA cloning and sequencing.

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EXAMPLE 1

Cloning of SCCA

1. 1. PCR amplification

mRNA from the cell-lines Caski (cervix), C4-I (cervix), A549 (lung), CaLu3 (lung), SkMes
 30 (lung), and RPMI2650 (pharynx) was prepared using QuickPrep Micro mRNA Purification
 kit (Pharmacia) and cDNA was prepared using First-Strand cDNA Synthesis kit
 (Pharmacia). A 1218bp DNA fragment covering the coding sequence of SCCA was
 amplified by PCR in a 100 µl reaction containing 10 mM Tris-HCl pH 8.85, 25 mM KCl, 5
 mM (NH₄)₂SO₄, 2 mM MgSO₄ (Boehringer), 0.2mM dNTP (Pharmacia), 10 µM SCCA 1-7F
 35 (DNA sequences for all primers are shown in Table 1), 10 µM SCCA 391-397B, 2 µl cDNA

and 2.5 U Pwo-polymerase (Boehringer). After denaturing samples for 5 min at 96°C a total of 30 cycles were performed, each consisting of denaturation for 15 sec at 96°C, annealing for 15 sec at 60°C, and extension for 30 sec at 72°C. The PCR reaction was completed by a final extension for 10 min at 72°C.

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TABLE 1. PCR-primers

	<u>Primer name</u>	<u>Sequence</u>
	1. SCCA 1-7F	5'-CGGGATCCATGAATTCAGTCAAGCC-3' (SEQ ID NO: 3)
10	2. SCCA 391-397B	5'-GAGCTCGAGTCTCATCAGTGACAGACTAATTGCATCTA-3' (SEQ ID NO: 4)
	3. SCCA 266-273F	5'-TGAATGGACAAGTTTGCAG-3' (SEQ ID NO: 5)
	4. SCCA1 323-329B	5'-GTAGGACTCCAGATAGCAC-3' (SEQ ID NO: 6)
	5. SCCA2 319-324F	5'-TGGAGCCACGGTCTCTCAG-3' (SEQ ID NO: 7)
15	6. SCCA2 357-363B	5'-ATTAGTTGAAGGAGATGATAATTC-3' (SEQ ID NO: 8)
	7. SCCA1 ex7	5'-AATACATACAAGTCCA-3' (SEQ ID NO: 9)
	8. SCCA2 ex8	5'-GGACTTTAGATACTGA-3' (SEQ ID NO: 10)

20 1. 2. Detection of SCCA1 and SCCA2

Presence of SCCA1 in PCR products were detected by cleavage with restriction enzyme SacII, resulting in two fragments, 245 and 973 bp, respectively, or by SCCA1-specific PCR using the primers SCCA1-7F and SCCA1 323-329B in a standard PCR reaction (75 mM Tris-HCl pH 8.8, 20 mM (NH₄)₂SO₄, 0.01% Tween 20, 2 mM MgCl₂, 0.2 mM dNTP, 10
25 μM of each primer, template, and 0.025 U/μl reaction Taq Polymerase; after denaturing samples for 5 min at 96°C a total of 30 cycles were performed, each consisting of denaturation for 15 sec at 96°C, annealing for 15 sec at optimal annealing temperature, and extension for 30 sec at 72°C. The PCR reaction was completed by a final extension for 10 min at 72°C.), Ta=50°C, resulting in a 997 bp fragment. Presence of SCCA2 were
30 detected by standard PCR using SCCA 1-7F and an SCCA2-specific primer, SCCA2 357-363B, Ta=60°C, giving a 1090 bp fragment.

1. 3. Cloning

PCR-products were cloned using PCR-Script Amp cloning kit (Stratagene). Colony
35 screening was performed by PCR as described in 1.2 above. Plasmid-DNA was prepared from selected clones containing SCCA1 or SCCA2 using Wizard Plus Minipreps DNA Purification System (Promega).

1. 4. DNA sequencing

Clones were sequenced using ABI Prism BigDye Terminator Cycle Sequencing (PE Biosystems). Samples were run on an ABI Prism 310.

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1. 5. Recloning

Selected clones were recloned into the expression vector pGEX-6P-3 (Pharmacia). Fragments were excised from the PCR-Script Amp vector using BamHI and XhoI and ligated into the expression vector in a 10 µl reaction containing 1xOPA, 1 mM ATP, 50 ng cleaved vector, SCCA insert corresponding to a moles-of-ends vector: insert ratio of 1:5-1:8, and 7.5-10 U T4DNA ligase (all from Pharmacia). Reaction tubes were incubated at 10°C overnight and inactivated for 10 min at 65°C. 2-4 µl of the reaction was transformed into E.coli JM109 (46). Plasmid-DNA from selected clones was then transformed into E.coli BL21 for protein expression.

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1. 6. Maintenance of cloned gene

Plasmid-DNA (pGEX-6P-3 containing the SCCA1/A2 fusion gene) in a 10 mM Tris-HCL pH 8.0 buffer solution is stored in -80°C. For resuming protein expression, plasmid-DNA ~~are~~ is transformed into competent E.Coli BL21 according to Sambrook et al. (p 1.82-1.84 in Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY*). For preparation of more plasmid-DNA, transformation into E. Coli JM109 is preferred.

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EXAMPLE 2

25 *Protein expression and purification*

2. 1. Protein Expression

Expression conditions were determined by small-scale preparations. For large scale expression 500 ml cultures of 2xYT and 100 µg/ml ampicillin were inoculated with 5 ml over-night culture and grown at 37°C. Protein expression was induced at OD₆₀₀=0.5-1.3 by adding IPTG to a final concentration of 0.1 mM. Cultures producing SCCA1 were grown for 4-16 h, SCCA1/A2 for 16-18 h. Cultures producing the SCCA2 protein were induced at OD₆₀₀= 1.2-1.4 and were grown for 2-3 h.

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2. 2. Protein Purification

Cells were harvested by centrifugation for 10 min at 2000 g, washed with 50 ml TE pH 8.0, and dissolved in 3 ml TE/g bacterial pellet. Lysozyme was added to a final concentration of 800 µg/g pellet and the mixtures were incubated on ice for 30-60 min and then frozen over night at -70°C. Magnesium chloride and DNase were added to a final concentration of 12 mM and 20 µg/g pellet, respectively. After incubation on ice for 30 min, samples were centrifuged for 30 min at 40000 g. To each supernatant 0.5 ml of 50% Glutathione Sepharose (Pharmacia) was added and incubated for 30 min-2 h at room temperature with gentle agitation. The slurry was washed 5-7 times using 1xPBS. GST-SCCA fusion protein was eluted using 0.5-1 ml Reduced Glutathione (Pharmacia) and incubated for 30-60 min at room temperature or over-night at 4°C, all with gentle agitation. SCCA protein was eluted by cleavage in between GST and SCCA. 0.48 ml cleavage buffer (50 mM Tris-HCl pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT) and 20µl PreScission protease were added and samples were incubated at 4°C with gentle agitation for 4 h or over-night. Proteins were analyzed on SDS-PAGE by Phast-system (Pharmacia).

2 3. Complex binding

Complex binding of SCCA to substrates was performed by mixing 2 µg of SCCA-protein with 0.5 µg of Cathepsin G (Bioscience Int.) or 0.5 µg of 0.9 µg Cathepsin L (Calbiochem) in 1xPBS buffer in a total volume of 4.5 µl. Samples were incubated at 37°C for 30 minutes. To each sample, 0.5 µl of 10xComplex-buffer (20% SDS, 140 mM Mercaptoethanol, bromophenolblue) was added. Samples were incubated for 3 minutes at 95°C and analyzed on a 12.5% SDS-PAGE-gel. The SCCA1/A2 fusion protein forms a complex with Cathepsin G but not with Cathepsin L showing that the fusion protein is functional and has the substrate specificity of SCCA2.

EXAMPLE 3

DNA analysis

3. 1. Southern Blot Analysis

Approximately 10 µg of DNA prepared from SCC cell-lines as well as from blood samples from normal healthy volunteers, were digested with restriction endonucleases PstI or BamHI. Digested DNA were separated on 0.8 % agarose and transferred to membranes (Hybond N+, Pharmacia). Filters were prehybridized for 1 h and hybridized over night at 60°C in 20 ml of a solution containing 5xSSC, 0.1% SDS, 5% Dextrane sulfate, Liquid

block (Pharmacia) diluted 1:20 and salmon sperm DNA 100µg/ml. Probe concentration during hybridization was 10 ng/ml. After hybridization filters were stringency washed for 15 min in 1xSSC/0.1%SDS and for 15 min in 0.2xSSC/1%SDS, both at 60°C. Probe hybridization was detected using Gene Images CDP-Star detection module (Pharmacia) with minor modifications. Filters were blocked for 1 hour at room temperature in a solution containing liquid block diluted 1:7.5. Then they were incubated in buffer A (0.1M Tris, 0.3M NaCl, pH 9.5) / 0.5% BSA for 15 min before adding the anti-fluorescein HRP conjugate diluted 1:6800 and then incubated for another 45 min. Filters were washed for 3x10 min in buffer A/0.3% Tween 20 before adding detection reagent. Filters were incubated for 2 min, washed briefly in 2xSSC and wrapped in plastic film. Hyperfilm MP was exposed for 35 min.

3. 2. Hybridization probes

Probes were generated and labeled by PCR in a reaction containing 60 µm each of dATP, dCTP, and dGTP, 24 µM dTTP, 40 µM Fluorescein-11-dUTP, 2 mM MgCl₂, 3µM forward primer, 3 µM backward primer, 15 ng DNA template (SCCA2-containing plasmid), 1 U Taq polymerase and 1xPCR buffer (Advanced Biotechnologies). Probe I: A 393 bp fragment of exon 8 (nucleotide 802-1194), primers SCCA 266-273F and SCCA 391-397B, Ta=50°C; Probe II: A 126 bp fragment of exon 8 (nucleotide 957-1082), primers SCCA2 319-324F and SCCA2 357-363B, Ta=50°C; probe III: A 1194 bp fragment covering the coding sequence and 22 nucleotides in the 3'-end of the gene, primers SCCA 1-7F and SCCA 391-397B, Ta=60°C.

Southern blot of PstI digested DNA hybridized with probe I show a different band pattern of DNA from a SCC-cell line compared to that of normal control DNA. DNA digested with BamHI also shows aberrant bands compared to normal control DNA.

3. 3. PCR analysis

DNA isolated by routine procedures from samples analysed by PCR using primers 7 and 8 (see Table 1) in a standard PCR-reaction show only product in samples containing the fusion gene.

EXAMPLE 4

Hybridomas and monoclonal antibodies

4. 1. Establishment of hybridomas and production of monoclonal antibodies reactive with SCCA1/A2, SCCA2 and SCCA1

Polyclonal antisera reactive with SCC antigen was obtained by subcutaneous immunization of Rabbits with recombinant SCC antigen and collection of immune sera according to standard procedures. The titer of the polyclonal antisera was tested by determination of the reactivity of the antisera with biotinylated SCCA1/A2 and SCCA1 immobilized in streptavidin plates (Labsystems Oy, Helsinki, Finland), Figure 6. The recombinant SCCA1/A2 and SCCA1 were biotinylated with Biotin-N-succinimide caproate ester according to standard procedures.

Monoclonal antibodies reactive with SCCA1/A2 and SCCA2 were established by immunization of Balb/c mice intra peritoneally with 10 - 50 µg of recombinant SCCA1/A2 in Ribi adjuvant. After the immunization and 2 - 4 booster doses during 60 - 90 days spleen cells from the immunized mice were fused with P3 x 63Ag 8 myeloma cells as described (47).

Hybridomas producing antibodies reacting with SCCA1/A2 were selected by ELISA screening of hybridoma supernatants in microtiter wells coated with affinity purified polyclonal antiserum against mouse IgG + M, (Jackson Immuno Res Lab, US). The wells were then incubated with SCCA1/A2 antigen, and after washing the bound antigen was detected by incubation with polyclonal Rabbit Anti SCC and HRP labeled Swine Anti Rabbit Ig (Dako AS, Copenhagen, Denmark).

4. 2. Reactivity of selected hybridomas with SCC antigens

The reactivity of the established hybridomas was tested in an ELISA similar to the ELISA screening procedure. Briefly the monoclonal antibodies produced by the hybridomas were immobilized in microtiter plates coated with polyclonal antiserum against mouse IgG+M (Jackson Immuno Res Lab, US). The wells were then incubated with 50 µL of the different recombinant SCC antigens in PBS 1% BSA for 1 h, after washing the plates were incubated with 100 µL Rabbit antiSCC diluted 1/5000 in PBS-1%BSA and incubated for additional 1h. The bound Rabbit Anti-SCC was then detected by incubation with HRP - Swine anti Rabbit Ig and visualized with OPD substrate and determination of OD at 450 nm.

In figure 7 the reactivity of selected hybridomas are shown. The SCC106, SCC114, SCC115 reacted only with SCCA1/A2, which indicate that they are specific for the SCCA1/A2 fusion protein. The SCC100, SCC103 and SCC109 reacted with SCCA2 and SCCA1/A2 but not with SCCA1 indicating that they are specific for SCCA2. The SCC110, SCC111 and SCC124 reacted with SCCA1 and SCCA1/A2 but not with SCCA2 suggesting that they are specific for SCCA1.

The SCC107, SCC119 and SCC128 reacted with all SCC antigens suggesting that they recognize a common epitope in SCCA1 and SCCA2.

Twice limiting dilution cloned clones producing antibodies reacting with SCCA1/A2, but negative for SCCA1 were produced.

Monoclonal antibodies were produced by in vitro cultivation of the hybridoma clones by inoculation of 10^4 cells/mL in DMEM, 5 % Fetal Calf Serum in roller bottles and allowed to grow for 10 - 14 days. The monoclonal antibodies were then purified from the culture medium by Protein A (Bioprocessing Ltd, Durham, UK) affinity chromatography according to the manufacturers recommendation.

EXAMPLE 5

Using the established monoclonal antibodies and recombinant proteins it was possible to develop immunoassays for specific determination of SCCA1/A2 fusion protein and assays specific for SCCA2 and SCCA1 respectively.

5. 1 Immunoassays for determination of SCCA1/A2 fusion protein

Assays specific for SCCA1/A2 fusion protein but essentially negative for SCCA1 and SCCA2 were designed by using antibodies among SCC106, SCC114 or SCC115 in combination with antibodies among SCC107, SCC119 or SCC128, see figure 7.

In the preferred configuration antibody SCC107 was used as catching antibody and SCC106 as detecting antibody.

SCC107 MAb was biotinylated with BiotinNHRS caproate ester, Sigma Chemical Co, US, using standard procedures, and used as catching antibody. SCC106 MAb were conjugated with HRP according to a modification of the Nakone procedure.

The biotinylated SCC107 MAb and HRP conjugated SCC106 MAb were used in two-site EIA according to the following protocol. Assay procedure

1. Add 50 µL of SCCA recombinant antigen (0 - 100 µg/L in PBS, 60 g/L BSA, pH 7.2)
5 + 100 µL of Biotin SCC107 MAb, 2 µg/mL, in Assay Buffer in Streptavidin coated microtiter plates, Labsystems Oy, Helsinki, Finland.
2. Incubate for 1 h ± 10 min with shaking
3. Wash 3 times with 5 mM Tris buffer, 0.05 % Tween 40, pH 7.75.
4. Add 100 µL HRP SCC106 MAb, 2 µg/mL, in Assay Buffer.
- 10 5. Incubate for 1 h ± 10 min with shaking.
6. Wash 6 times with 5 mM Tris buffer, 0.05 % Tween 40, pH 7.75.
7. Add 100 µL TMB, ELISA Technology, US.
8. Incubate 30 min ± 5 min
9. Determine OD 620 nm in ELISA reader.

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Dose-response curves for SCCA1, SCCA2 and SCCA1/A2 antigens revealed that the assay was specific for the SCCA1/A2 recombinant antigen with < 5 % cross reactivity with SCCA1 or SCCA2.

20 5. 2 Assays for specific determination of SCCA2

Assays specific for SCCA2 without significant reactivity with SCCA1/A2 and SCCA1 were designed by using antibodies among SCC100, SCC103 or SCC109 in combination with antibodies among SCC107, SCC119 or SCC128. In the preferred configuration SCC107 MAb was used as catching antibody and the SCC103 was used as detecting antibody.

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SCC107 MAb was biotinylated with BiotinNHRS caproate ester (Sigma Chemical Co, US) using standard procedures, and used as catching antibody. SCC103 MAb was conjugated with HRP, Type V (Sigma Chemical Co, US), according to a modification of the Nakone procedure.

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The biotinylated SCC107 MAb and HRP conjugated SCC103 MAb were used in two-site EIA according to the following protocol.

Assay procedure:

1. Add 50 µL of SCC recombinant antigen (0 - 100 µg/L in PBS, 60 g/L BSA, pH 7.2) +

100 µL of Biotin SCC107 MAb, 2 µg/mL, in Assay Buffer in Streptavidin coated microtiter plates (Labsystems Oy, Helsinki, Finland).

2. Incubate for 1 h ± 10 min with shaking
3. Wash 3 times with 5 mM Tris buffer, 0.05 % Tween 40, pH 7.75.
- 5 4. Add 100 µL HRP SCC103 MAb 2 µg/mL, in Assay Buffer.
5. Incubate for 1 h ± 10 min with shaking.
6. Wash 6 times with 5 mM Tris buffer, 0.05 % Tween 40, pH 7.75.
7. Add 100 µL TMB, ELISA Technology, US
8. Incubate 30 min ± 5 min
- 10 9. Determine OD 620 nm in ELISA reader.

Based on the dose-response curves for SCCA2, SCCA1 and SCCA1/A2 fusion protein it was concluded that the assay according to example 5.2 was specific for SCCA2 with a cross-reactivity of < 5 % for SCCA1 and SCCA1/A2.

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5. 3. Assays for specific determination of SCCA1

Assays specific for SCCA1 without significant reactivity with SCCA2 and SCCA1/A2 were designed by using antibodies among SCC110, SCC111 or SCC124 in combination with antibodies of among SCC107, SCC119 or SCC128. In the preferred configurations

20 SCC107MAb was used as catching antibody and SCC124 MAb was used as detecting antibody.

SCC107 MAb was biotinylated with BiotinNHRS caproate ester (Sigma Chemical Co, US) using standard procedures, and used as catching antibody. SCC124 MAb was conjugated

25 with HRP, Type V, (Sigma Chemical Co., US) according to a modification of the Nakone procedure.

The biotinylated SCC107 MAb and HRP conjugated SCC124 MAb were used in two-site EIA according to the following protocol.

30 Assay procedure

Add 50 µL of SCC antigen (0 - 100 µg/L in PBS, 60 g/L BSA, pH 7.2)

+ 100 µL of Biotin SCC107 MAb, 2 µg/mL, in Assay Buffer in Streptavidin coated microtiter plates (Labsystems Oy, Helsinki, Finland).

2. Incubate for 1 h ± 10 min with shaking
- 35 3. Wash 3 times with 5 mM Tris buffer, 0.05 % Tween 40, pH 7.75.

4. Add 100 μ L HRP SCC124 MAb, 2 μ g/mL, in Assay Buffer.
5. Incubate for 1 h \pm 10 min with shaking.
6. Wash 6 times with 5 mM Tris buffer, 0.05 % Tween 40, pH 7.75.
7. Add 100 μ L TMB, (ELISA Technology, US).
- 5 8. Incubate 30 min \pm 5 min
9. Determine OD 620 nm in ELISA reader.

Based on the antibodies according to 5.3 immunoassays specific for SCCA1 with < 10 % cross-reactivity for SCCA2 or SCCA1/A2 antigen may be designed.